FORM PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: GJE-247

U S APPLICATION NO (If known, see 37 CFR 1 5): 09/914667

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/01198	29 March 2000	31 March 1999

TITLE OF INVENTION: Biocatalyst And Its Use In Enzymatic Resolution Of Racemic Beta-Lactams

APPLICANT(S) FOR DO/EO/US: Stephen John Clifford Taylor and Philip Alexander Keene

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☑ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☑ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☑ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☑ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), executed
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 37 CFR 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/914667	PCT/GB00/01198	GJE-247

21. ☑ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	18 - 20 =	0	x **$18.00**		
Independent claims	2 - 3 =	0	x **$80.00**		
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**		
TOTAL OF ABOVE CALCULATIONS =				$860.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +					
SUBTOTAL =				$860.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE =				$860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					
TOTAL FEES ENCLOSED =				$860.00	
				Amount to be refunded:	$
				charged:	$

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☑ Please charge my Deposit Account No. 19-0065 in the amount of $ 860.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0065 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

CORRESPONDENCE ADDRESS:

CUSTOMER NUMBER
23,557

David Saliwanchik
SIGNATURE

August 31, 2001
DATE

David R. Saliwanchik
NAME

31,794
REGISTRATION NUMBER

August 31, 2001

Patent Application
Docket No. GJE-247

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Stephen John Clifford Taylor and Philip Alexander Keene

Docket No. : GJE-247

For : Biocatalyst And Its Use In Enzymatic Resolution Of Racemic Beta-Lactams

PRELIMINARY AMENDMENT

Please amend the above-identified patent application as follows:

In the Claims

Please amend the claims to read as follows:

Claim 1 (amended):

1. A process for the preparation of an enantiomerically enriched β-lactam, which comprises enantioselective hydrolysis of the corresponding racemic β-lactam in the presence of a lactamase enzyme capable of enantioselective hydrolysis of 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en4-one and 7-azabicyclo[4.2.0]oct-4-en-8-one.

Claim 2 (amended):

The process according to claim 1, wherein the lactamase enzyme is in isolated and purified form.

Claim 3 (amended):

The process according to claim 1, wherein in the lactamase enzyme is in the form of a cell paste or intact cells.

Claim 4 (amended):

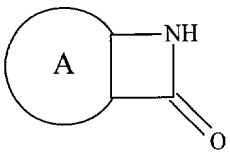
The process according to claim 1, which additionally comprises isolation of the enantiomerically enriched β-amino acid produced by hydrolysis.

Claim 5 (amended):

The process according to claim 4, wherein the isolated β-amino acid is then subjected to a condensation reaction to reform the β-lactam ring.

Claim 6 (amended):

The process according to claim 1, wherein the lactam is a fused polycyclic compound of the type represented by formula (1)

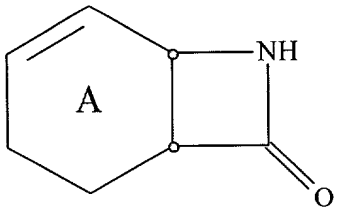


(1)

wherein ring A is any monocyclic or any polycyclic ring, optionally substituted with one or more non-interfering groups.

Claim 7 (amended):

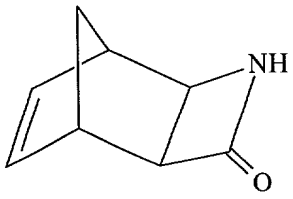
The process according to claim 6, wherein the lactam has the formula



wherein ring A is unsaturated and optionally also bridged or further fused.

Claim 8 (amended):

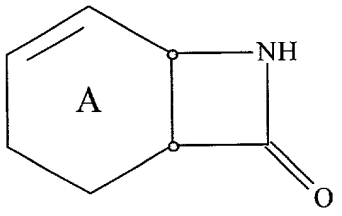
The process according to claim 7, wherein the lactam is 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one (2)



(2)

Claim 9 (amended):

The process according to claim 1, wherein the lactam is 7-azabicyclo[4.2.0]oct-4-en-8-one (3)



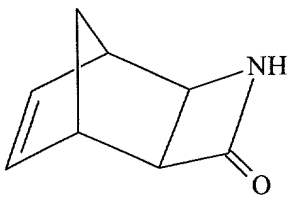
(3)

Claim 10 (amended):

The process according to claim 1, wherein the lactamase enzyme is obtainable from a microorganism having the characteristics of that available as the *Rhodococcus globerulus* strain identified as CMC103381, Accession No. NCIMB 41042.

Claim 11 (amended):

An enantiomerically enriched 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one of formula (2)

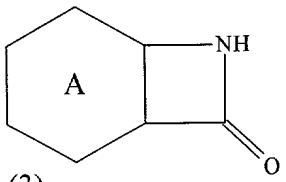


(2)

in an enantiomeric excess of at least 80%.

Claim 12 (amended):

An enantiomerically enriched 7-azabicyclo[4.2.0]oct-4-en-8-one of formula (3)



(3)

in an enantiomeric excess of at least 80%.

Claim 13 (amended):

A lactam according to claim 11, wherein the enantimeric excess is at least 95%.

Claim 14 (amended):

The enantimerically enriched enantiomer according to claim 11 in the levorotatory form.

Please add the following new claims:

17. The lactam according to claim 12, wherein the enantiomeric excess is at least 95%.

18. The enantimerically enriched enantiomer according to claim 12, in the levorotatory form.

Remarks

Claims 1-14 have been amended and new claims 17 and 18 have been added.

No new matter has been added by these amendments.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

Respectfully Submitted

David Saliwanchik

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Phone No.: 352-375-8100
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Suite A-1 Gainesville, FL 32606

DRS/la
Attachment: Marked-up Version of Amended Claims

Marked-up Version of Amended Claims

Claim 1 (amended):

1. ~~Process~~ A process for the preparation of an enantiomerically enriched β-lactam, which comprises enantioselective hydrolysis of the corresponding racemic β-lactam in the presence of a lactamase enzyme capable of enantioselective hydrolysis of 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en4-one and 7-azabicyclo[4.2.0]oct-4-en-8-one.

Claim 2 (amended):

~~A~~ The process according to claim 1, wherein the lactamase enzyme is in ~~the form of a~~ isolated and purified form.

Claim 3 (amended):

~~A~~ The process according to claim 1, wherein in the lactamase enzyme is in the form of a cell paste or intact cells.

Claim 4 (amended):

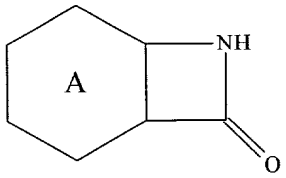
~~A~~ The process according to ~~any preceding claim~~ claim 1, which additionally comprises isolation of the enantiomerically enriched β-amino acid produced by hydrolysis.

Claim 5 (amended):

~~A~~ The process according to claim 4, wherein the isolated β-amino acid is then subjected to a condensation reaction to reform the β-lactam ring.

Claim 6 (amended):

~~A~~ The process according to ~~any preceding claim~~ claim 1, wherein the lactam is a fused polycyclic compound of the type represented by formula (1)



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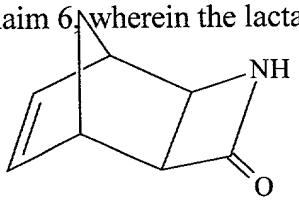
wherein ring A is any monocyclic or any polycyclic ring, optionally substituted with one or more non-interfering groups.

Claim 7 (amended):

~~A~~ The process according to claim 6, wherein the lactam has the formula



wherein ring A is unsaturated and optionally also bridged or further fused.

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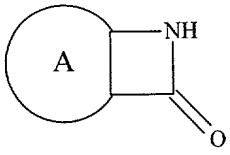
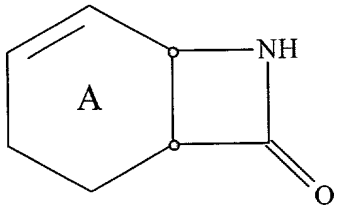
~~A~~ The process according to claim 7, wherein the lactam is 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one (2)



(2)

Claim 9 (amended):

~~A~~ The process according to claim 1, wherein the lactam is 7-azabicyclo[4.2.0]oct-4-en-8-one (3)



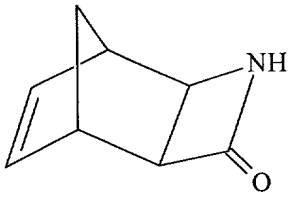
(3)

Claim 10 (amended):

~~A~~ The process according to ~~any preceding claim~~ claim 1, wherein the lactamase enzyme is obtainable from a microorganism having the characteristics of that available as the *Rhodococcus globerulus* strain identified as CMC103381, Accession No. NCIMB 41042.

Claim 11 (amended):

An enantiomerically ~~Enantiomerically~~ enriched 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one of formula (2) [as shown in claim 8,]

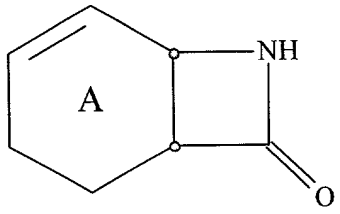


(2)

in an enantiomeric excess of at least 80%.

Claim 12 (amended):

An enantiomerically ~~Enantiomerically~~ enriched 7-azabicyclo[4.2.0]oct-4-en-8-one of formula (3) ~~as shown in claim 9,~~



(3)

in an enantiomeric excess of at least 80%.

Claim 13 (amended):

A lactam according to claim 11 ~~or claim 12~~, wherein the enantimeric excess is at least 95%.

Claim 14 (amended):

The enantimerically ~~Enantimerically~~ enriched ~~levorotatory~~ enantiomer according to ~~any of claims 11 to 13~~ claim 11 in the levorotatory form.

BIOCATALYST AND ITS USE IN ENZYMATIC RESOLUTION OF RACEMIC BETA-LACTAMS

Field of the Invention

This invention relates to a process for the production of optically active β-lactams by enzymatic resolution of the racemic β-lactam.

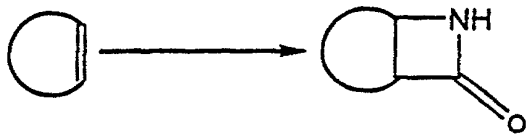
Background of the Invention

The utility of the β-lactam ring as a structural feature in biologically important compounds is well established, as is the synthetic utility of β-lactams. For example, the utility of β-lactams as masked β-amino acids is illustrated by their use as precursors to the C-13 taxol side chain.

The active molecules are often chiral, and it is preferable that they are accessible in the form of single enantiomers. This feature is also utilised in the design of stereodefined scaffolds, to generate single enantiomer compounds libraries for initial lead identification as part of a drug discovery programme.

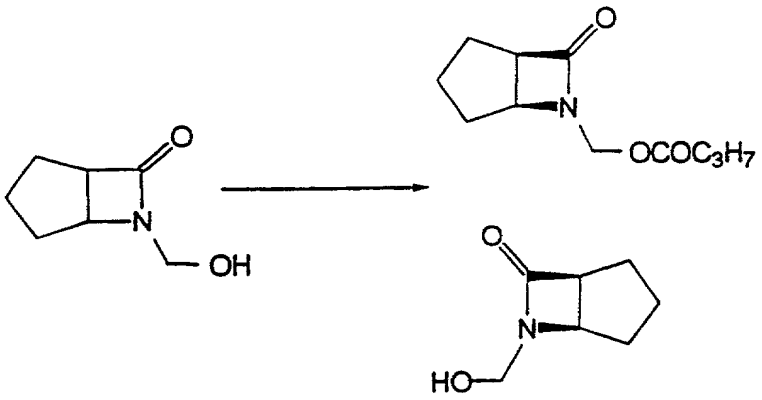
Alicyclic β-amino acids can be cyclised to the corresponding β-lactams, typically by use of carbodiimide reagents. They are also compounds having biological or pharmaceutical activity. For example, the natural product cispentacin, (-)-(1*R*,2*S*)-2-aminocyclopentanecarboxylic acid, has potent antifungal properties. It is synthesised as a natural product in *Streptomyces setonii* and *Bacillus cereus*, and is inhibitory to strains of *Candida*. The same amino acid has also been used to probe the relationship between structure and taste in L-aspartyl dipeptides, where the absolute structure of the β-amino acid strongly affected the taste of the dipeptide.

Optically pure alicyclic β-amino acids may be made, for example, by the enzyme-catalysed solvent-based bioresolution of the amino carboxyl esters (Kanerva *et al.*, *Tetrahedron: Asymmetry*, 1996, 7, 1705). Such racemic amino esters are readily synthesised from β-lactams of formula (1), which are themselves synthesised by cycloaddition of the appropriate cyclic alkene and chlorosulfonyl isocyanate, followed by reductive work-up using sodium sulfite:

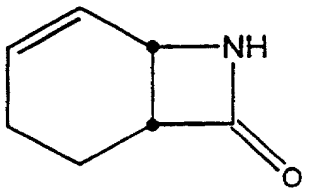


(1)

It is generally preferable to resolve enantiomers as early as possible in a synthetic sequence, to avoid having to perform the chemistry on the racemate at twice the scale as for the enantiomer. Thus, it is useful to be able to access β-lactam precursors of β-amino acids in resolved form. One approach is to derivatise the β-lactam to the *N*-hydroxymethyl-β-lactam using paraformaldehyde (Csomós *et al.*, *Tetrahedron: Asymmetry*, 1996, 7, 1789). The resulting primary alcohols can then be resolved using a lipase, for example to access the *O*-acylated precursor to cispentacin, as depicted below. Besides the extra derivatisation and deprotection required, the resolution with lipase AK requires high enzyme loading and chromatographic separation of the products, making this general approach unattractive at scale.



WO-A-97/10713 and EP-A-0232017 disclose various bicyclic compounds, including 7-azabicyclo[4.2.0]oct-4-en-8-one of the formula

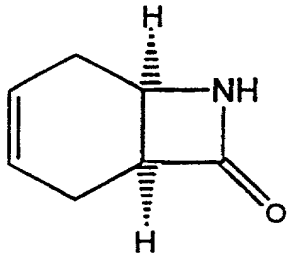


(3)

These compounds are in racemic form. WO-A-97/10713 includes reference to enantiomers, but there is no evident means of resolution. For example, the compounds will not readily form salts with chiral resolving agents.

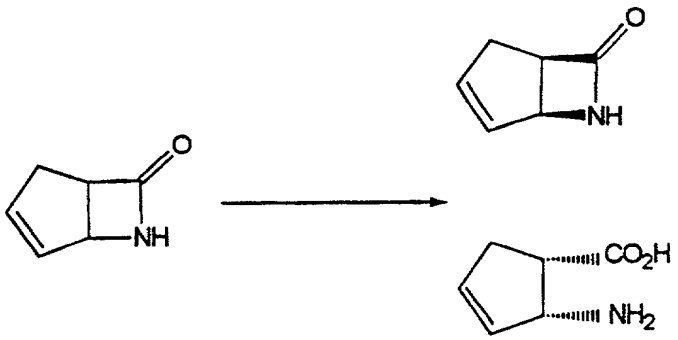
EP-A-0002564 discloses β-lactams, including bicyclic structures. It is suggested that the racemic lactams might be resolved, and refers to GB-A-1273278, but that depends on melt crystallisation, and probably requires that the substrate exists as a conglomerate.

Kurihara *et al*, Tetrahedron Lett., 1985, **26** (47), 5831, and also Tamara *et al*, Tetrahderon Lett. 1986, **27** (32), 3749, disclose a β-lactam, in single enantiomer form, of the formula



In the former paper, enzymatic desymmetrisation of a precursor (before conversion to a β-amino acid and then formation of the lactam ring) is involved. The latter involves classical resolution, using cinchonidine, of a ring-open precursor.

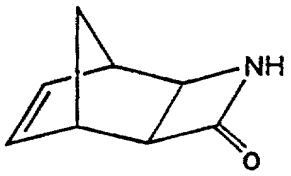
A more direct approach would be to resolve the lactam itself, thereby removing the separation problem evident in the work of Csomós *et al* (reference cited above), since the resulting amino acid is soluble in buffer, whilst the unreacted substrate can be extracted into solvent. This has been shown in one instance (Evans *et al*, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2276; see also WO-A-92/18477) where *Rhodococcus equi* was used to provide (1*R*, 5*S*)-6-azabicyclo[3.2.0]hept-3-ene-7-one:



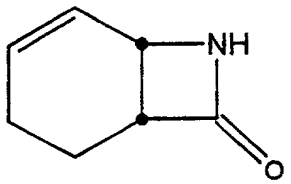
However, this biotransformation is an exceedingly slow process and is therefore not viable for operation on a commercial scale; 980mg whole-cell paste was needed to resolve 340mg racemate in 212 hrs, in order to recover unreacted lactam of >99% ee.

Summary of the Invention

The present invention is based on the discovery of a novel lactamase biocatalyst that allows efficient access to a range of synthetically useful β-lactams and the corresponding β-amino acids. A preferred embodiment of the invention is the application of this methodology to the preparation of novel single enantiomer cyclohexene-fused β-lactams. More particularly, the novel biocatalyst is capable of enantioselective hydrolysis of racemic lactams of the formulae (2) and (3)



(2)



(3)

A further aspect of the invention lies in the novel, enantiomerically enriched compounds of formulae (2) and (3), preferably as the levorotatory enantiomer.

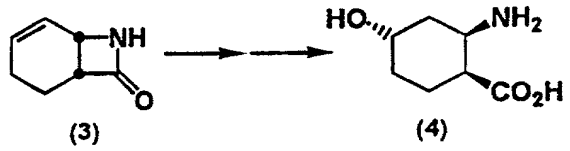
Description of the Invention

For identification of a suitable biocatalyst, a microbial screen was targeted at hydrolysis of racemic lactams (2) and (3). Hitherto, neither compound has been prepared in enantiomerically enriched form. Lactams (2) and (3) are conveniently synthesised by cycloaddition of chlorosulfonyl isocyanate with norbornadiene and cyclohexa-1,3-diene respectively (Stájer *et al.*, *Tetrahedron*, 1984, **40**, 2385; Malpass *et al.*, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2276).

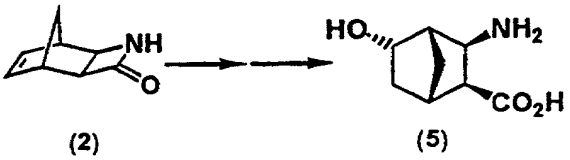
400 Microbial strains were screened for hydrolysis of the lactams (2) and (3). Surprisingly, one strain of *Pseudomonas putida* not only hydrolysed the lactam, but did so selectively, with much higher activity than observed for the prior art strain of *Rhodococcus* (Evans *et al*, reference as above). Thus, for lactam (2), when stirred with an equal weight of cell paste in phosphate buffer at 25°C, resolution was complete after 15 hours (>98% ee residual substrate), and the enantiomerically pure lactam extracted into

ethyl acetate in 38% yield. Similarly for lactam (3), after incubation with an equal weight of cells for 21 hours in buffer, the ee of residual lactam was >90%. Recovery of the enantiomerically enriched β-amino acids from aqueous solution is facilitated by conversion to *N*-Boc derivatives under standard conditions, followed by solvent extraction.

Enantiomerically enriched products obtained from the bioresolution of lactams (2) and (3) can be used to prepare information-rich chiral scaffolds for elaboration into single enantiomer compounds libraries. The alkene functionality is amenable to a range of transformations, especially through reaction with oxidants. For example, conversion of (3) to the chiral scaffold (4) gives three points for structural elaboration into defined regions of 3-dimensional space. Similarly, the more rigid scaffold (5) can be prepared via bioresolution of (2).



(3) (4)



(2) (5)

In summary, the present invention embodies a novel β-lactamase biocatalyst from *Pseudomonas putida*, combining the key attributes of high enantioselectivity with superior catalytic activity to β-lactamases described previously. This biocatalyst has been deposited (see Example 1). Use of this biocatalyst allows certain single enantiomer β-lactams to be prepared for the first time in synthetically useful amounts.

As will be evident to those skilled in the art, the biocatalyst can be used under conditions that can readily be determined, to produce a mixture of compounds. These can be separated by known methods. More particularly, the enantiomerically enriched β-amino acid produced by hydrolysis can be isolated. The isolated β-amino acid may then be subjected to a condensation reaction, to reform the β-lactam ring.

The biocatalyst can be used in whole cell or paste form. The enzyme can also be isolated, by techniques known to those skilled in the art.

The following Examples 1, 2, 4, 6 and 8 illustrate the invention. Examples 3, 5 and 7 illustrate the preparation of racemic substrates. TFAA is trifluoroacetic anhydride.

Example 1 **Biocatalyst**

Glycerol stocks of 96 bacterial strains (obtained from the Applicant's strain collection) were used to inoculate 1.0ml Tryptone soya broth (Oxoid CM129) per well in 2.2ml 96-well plates (Advanced Biotechnologies AB-0661). These were then shaken at 25°C on a Heidolph Titramax 1000 incubator at max rpm for 45 hours. The cells were harvested by centrifugation at 1000g, 4°C, for 10 minutes, and the cell pastes were stored at -20°C.

Cell pastes of the 96-well culture plates SCL0003 were resuspended in 0.5ml of 20g.L^{-1} of substrate in 0.1M KH_2PO_4, pH 7.0 (13.6g KH_2PO_4 in 1.0L water, adjusted to pH 7.0 with 12M NaOH) and then shaken at 25°C on a Heidolph Titramax 1000 incubator at max rpm for 40 (on the norbornadiene lactam (2) derivative) to 66 hours (on the cyclohexa-1,3-diene lactam (3) derivative). Reactions were stopped by diluting 1 in 10 in a 1:1 mix of MeOH:10mM KH_2PO_4, pH 7.0 (1.36g KH_2PO_4 in 1.0L water, adjusted to pH 7.0 with 12M NaOH). These were then assayed by HPLC. A 15cm 5µ Hichrom KR100 C8 Column was used with a running buffer of a 1:1 mix of MeOH:10mM KH_2PO_4, pH 7.0 (1.36g KH_2PO_4 in 1.0L water, adjusted to pH 7.0 with 12M NaOH) at a flow rate of 1.0ml.min^{-1}. Detection was at 210 nm. Results showed that a strain of *Pseudomonas putida* had reached 45% conversion on the norbornadiene lactam derivative and 43% conversion on the cyclohexa-1-3-diene lactam derivative. This strain, designated CMC 103381, was deposited at NCIMB Ltd., 23 St. Machar Drive, Aberdeen AB24 3RY, Scotland, on 31.03.99 and again on 29.03.00. The respective accession numbers are 41013 and .....

Example 2 **Cell Paste**

A 10µl loopful of colony of CMC 103381 (from agar plate) was used to inoculate seed flasks (100ml Tryptone soya broth (Oxoid CM129) per 500ml Erlenmeyer flask). These were shaken at 25°C, 300rpm in a temperature-controlled shaker (New Brunswick G-25 with 25 mm (1 inch) throw) for 23 hours. A 0.67% v/v inoculum was used to inoculate the fermenters. The fermenters used were 3.0l Applikon fermenters containing

1.5L medium per fermenter. The fermentation medium contained, per litre: 15g yeast extract (Oxoid L21), 8g KH_2PO_4, 7g K_2HPO_4, 1g $MgSO_4.7H_2O$, 1g $(NH_4)_2SO_4$, 1ml trace elements solution, 1.0ml polypropylene glycol (Merck 29767 6Y), and 20g glucose. The trace elements solution contained, per litre: 250ml conc. HCl, 3.6g $CaCl_2.2H_2O$, 2.0g ZnO, 0.85g $CuCl_2.2H_2O$, 2.0g $MnCl_2.4H_2O$, 5.4g $FeCl_3.6H_2O$, 2.4g $CoCl_2.2H_2O$, 4.8g $Na_2MoO_4.2H_2O$, and 0.3g H_3BO_3. Control was with Biolab II controllers (Brighton systems). The fermenters were grown at 25°C, pH control between 6.9 and 7.1 (with 5M $NaOH/H_3PO_4$), 800 rpm, air flow at 1.0ml.min^{-1} for 40 hours. Cell pastes were harvested by centrifugation at 9,000g, 4°C for 20 minutes and were stored at -20°C for future use.

Example 3 ***exo*-3-Aza-tricyclo[4.2.1.0^{2,5}]non-7-en-4-one**

A solution of 20.3g norbornadiene in 100ml dichloromethane was slowly added, with stirring, to a solution of 19.1ml chlorosulfonyl isocyanate in 40ml dichloromethane. Upon addition, the solution turned red and gradually darkened to a deep purple colour. After the addition was complete, the mixture was left stirring for a further 30 minutes. The solution was then slowly added to a stirred mixture of 75ml of 33% sodium sulfite solution and 50ml dichloromethane, keeping the temperature at 25°C. The reaction mixture was left stirring for a further 30 minutes after the addition was complete. The organic layer was then separated from the aqueous and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 18g, 61%, of a white solid.

GC-MS : indicated m/z: 135 (M^+), 107, 91, 70.

Example 4 **Bioresolution of *exo*-3-Azatricyclo[4.2.1.0^{2,5}]non-7-en-4-one**

Into a 125ml conical flask were placed 2g lactam, 40ml of 50mM phosphate buffer, pH 7 and 0.5g *P. putida* CMC103381. The reaction vessel was placed inside a heated jacket to ensure that the temperature remained at 25°C. The reaction mixture was gently stirred and, after 24 hours, a further 1.5g of *P. putida* cells were added to the reaction mixture. After a total reaction time of 39 hours, an aliquot from the reaction was found by chiral GC to contain only a single enantiomer of the cyclic β-lactam. The reaction was halted after 46 hours, the enzyme cells were spun out by centrifugation (3400 rpm) and the resulting pellet was washed with distilled water and respun. The supernatant was extracted with 2 x 70ml ethyl acetate, the organic extracts were combined and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 770mg of a

white solid, $[\alpha]_D = -91°$ (20°C, MeOH). Chiral GC analysis was carried out using the Chirasil Dex-CB column: Retention time: 4.77 minutes, enantiomeric excess >95%.

Example 5 **7-Azabicyclo[4.2.0]-oct-4-en-8-one**

A solution of 10ml of cyclohexa-1,3-diene in 35ml of dichloromethane was slowly added, with stirring, to a solution of 7.4ml of chlorosulfonyl isocyanate in 150ml of dichloromethane. Upon addition, the solution turned red and gradually darkened to a deep purple colour. After the addition was complete, the mixture was left stirring for a further 5 minutes. The solution was then slowly added to a stirred mixture of 100ml of 25% sodium sulfite solution and 50ml dichloromethane. The reaction mixture was left stirring for a further 20 minutes after the addition was complete. The organic layer was then separated from the aqueous and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 3.4g, 33%, of a yellow oil which solidified on standing.

GC-MS : indicated m/z: 123 (M^+), 94, 80

^{1}H NMR ($CDCl_3$): 6.3-5.8 (2H; m), 4.1 (1H; t), 3.5 (1H; brs), 2.3-1.3 (4H; m).

Example 6 **Bioresolution of 7-azabicyclo[4.2.0]-oct-4-en-8-one**

Into a 100ml conical flask were placed 1.49g lactam, 40ml of 50mM phosphate buffer, pH 7 and 1.41g *P. putida* CMC103381. The reaction vessel was placed within a heated jacket and the temperature was maintained at 30°C. The reaction mixture was stirred continuously for 22 hours, after which time an aliquot was taken and analysed by chiral GC to determine whether the resolution was complete. The aliquot was extracted with ethyl acetate and derivatised using TFAA. The resulting chiral GC chromatogram indicated lactam with an e.e. of 93%. The reaction was halted, enzyme cells were spun off by centrifuge (3400 rpm) and the supernatant was extracted with 3 x 50ml of ethyl acetate. The organic phase was dried over magnesium sulfate and solvent was removed by rotary evaporator to yield 460mg of an off-white solid, $[\alpha]_D = -105.5°$ (20°C, MeOH).

Chiral GC analysis was carried out using a Chirasil Dex-CB column: Retention times: 15.46 mins (major enantiomer), 15.79 mins (minor enantiomer). Enantiomeric excess: 93%.

The amino acid produced from the bioresolution of 7-azabicyclo[4.2.0]-oct-4-en-8-one was isolated in two steps as the hydrochloride salt.

The 15ml of buffered solution remaining from the bioresolution described above contain 1g (7.1mmol) of amino acid (maximum). To the stirred buffer solution was slowly

added, at 10C, 1.55g (7.1mmol) dibutyl dicarbonate dissolved in 20ml THF. The pH of the solution was maintained at 9 by addition of 3M NaOH solution. The reaction mixture was allowed to warm up to room temperature and left stirring overnight. The reaction was halted after 18 hours and THF was removed by rotary evaporator. The aqueous reaction mixture was acidified to pH3 with 10% potassium hydrogen sulfate solution. The mixture was then extracted with 3 x 50ml of ethyl acetate and the combined organic extracts were dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 1.1g, 62% of a white solid.

Into a 25ml conical flask was placed 0.71g (2.81mmol) of Boc-protected amino acid, dissolved in 15ml THF. The stirred reaction mixture was acidified to pH 3 with 3M HCl solution and the reaction was vigorously stirred for 3 hours at ambient temperature. The reaction mixture was left to stand overnight and extracted with 10ml ethyl acetate. The aqueous phase was concentrated under vacuum to yield 480mg, 87% yield of an off-white solid.

^{1}H NMR (CD_3OD): 6.05-5.85 (1H; m), 5.65-5.50 (1H; m), 3.9 (1H; brs), 2.85 (1H; dt), 2.15-1.65 (4H; m).

Example 7 **6-Azabicyclo[3.2.0]hept-3-en-7-one**

Cyclopentadiene was freshly prepared by thermolysis of cyclopentadiene dimer. It was stored at -20°C, until required to prevent dimerisation. A solution of cyclopentadiene (1.65g, 25mmol) in dichloromethane (15ml) was slowly added to a stirred solution of chlorosulphonyl isocyanate (1.8ml, 21mmol) in dichloromethane (35ml) at -20°C for 15 minutes. Upon the addition the solution turned to dark red. The temperature was kept -20°C. After a further 20 minutes, a solution of anhydrous sodium bisulfite (6g in 25ml water) was added dropwise to the stirred solution. After 30 minutes the organic layer was separated and the aqueous layer was extracted twice with dichloromethane (15ml). The combined organic phases were dried over magnesium sulfate then evaporated. 1H-NMR showed a 55/1 mixture of the 6-azabicyclo[3.2.0]hept-3-en-7-one and the 2-azabicyclo[2.2.1]hept-5-en-3-one. The products were separated by chromatography on silica gel. 6-Azabicyclo[3.2.0]hept-3-en-7-one was isolated as a yellow oil (0.95g, 40%).

Example 8 **Bioresolution of racemic 6-azabicyclo[3.2.0]hept-3-en-7-one**

In a jacketed vessel at 30°C were placed 10ml phosphate buffer (50 mM, pH 7), 200mg 6-azabicyclo[3.2.0]hept-3-en-7-one and *P. putida* CMC103381 (200mg). The

reaction was stirred overnight. The ee of the residual substrate was assayed by chiral gas chromatography using a TFAA-derivatised sample. An aliquot was taken after 22h, derivatised using TFAA and analysed by GC.

Chiral Gas Chromatography, column Chirasil Dex CB

Racemate : enantiomer **1** retention = 4.09 min, enantiomer **2** t = 4.40 min.

Sample of the bioresolution reaction : GC chromatogram revealed almost exclusively single enantiomer (enantiomer **1** t = 4.09 min), giving residual substrate of >98%ee.

CLAIMS

1. A process for the preparation of an enantiomerically enriched β–lactam, which comprises enantioselective hydrolysis of the corresponding racemic β–lactam in the presence of a lactamase enzyme capable of enantioselective hydrolysis of 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one and 7-azabicyclo[4.2.0]oct-4-en-8-one.

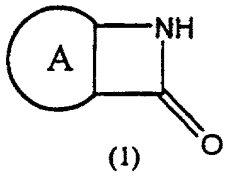
2. A process according to claim 1, wherein the lactamase enzyme is in isolated and purified form.

3. A process according to claim 1, wherein the lactamase enzyme is in the form of a cell paste or intact cells.

4. A process according to any preceding claim, which additionally comprises isolation of the enantiomerically enriched β-amino acid produced by hydrolysis.

5. A process according to claim 4, wherein the isolated β-amino acid is then subjected to a condensation reaction to reform the β-lactam ring.

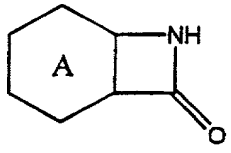
6. A process according to any preceding claim, wherein the lactam is a fused polycyclic compound of the type represented by formula (1)



(1)

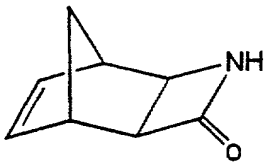
wherein ring A is any monocyclic or any polycyclic ring, optionally substituted with one or more non-interfering groups.

7. A process according to claim 6, wherein the lactam has the formula



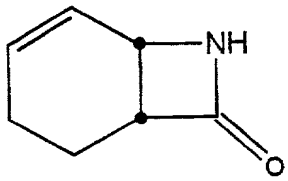
wherein ring A is unsaturated and optionally also bridged or further fused.

8. A process according to claim 7, wherein the lactam is 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one (2)



(2)

9. A process according to claim 1, wherein the lactam is 7-azabicyclo[4.2.0]oct-4-en-8-one (3)



(3)

10.(amended) A process according to any preceding claim, wherein the lactamase enzyme is obtainable from a microorganism having the characteristics of that available as the *Rhodococcus globerulus* strain identified as CMC103381, Accession No. NCIMB 41042.

11. Enantiomerically enriched 3-azatricyclo[4.2.1.$0^{2,5}$]non-7-en-4-one of formula (2) as shown in claim 8, in an enantiomeric excess of at least 80%.

12. Enantiomerically enriched 7-azabicyclo[4.2.0]oct-4-en-8-one of formula (3) as shown in claim 9, in an enantiomeric excess of at least 80%.

13. A lactam according to claim 11 or claim 12, wherein the enantiomeric excess is at least 95%.

14. Enantiomerically enriched levorotatory enantiomer according to any of claims 11 to 13.

15.(amended) A lactamase enzyme obtainable from a microorganism having the characteristics of that available as the *Rhodococcus globerulus* strain identified as CMC103381, Accession No. NCIMB 41042.

16.(amended) *Rhodococcus globerulus* strain identified as CMC103381, Accession No. NCIMB 41042.

USA

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of subject matter which is claimed and for which a patent is sought on an invention entitled

BIOCATALYST AND ITS USE IN ENZYMATIC RESOLUTION OF RACEMIC BETA-LACTAMS

S.T. MC

the specification of which ☐ is attached hereto or

☒ was filed on 29 MAR 2000 as United States Application Number or PCT International Application Number PCT/GB00/01198 and was amended on (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for a patent or inventor`s certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date	Priority Not Claimed	Certified Copy Attached? YES	NO
9907415.5	GB	29 MAR 1999	☐	☐	☐
			☐	☐	☐
			☐	☐	☐

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C 1001 and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

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